Zylkéne to Load? The effects of alpha-casozepine on compliance and coping in horses during loading.

Carrie Ijichi*[1,2], Sophie Green[1], Keith Squibb[1], Aisling Carroll[1] & Isobel Bannister[1]

Hartpury University, Hartpury, Gloucester, UK, GL19 3BE

[2]Present address: School of Animal, Rural and Environmental Science, Nottingham Trent University, Southwell, NG25 0QF

carrie.ijichi@ntu.ac.uk

Abstract

Horses are routinely travelled for access to safe off-road riding, veterinary care, breeding, sale or moving to a new home environment. However, transport is a known stressor in horses. For this reason, problem behaviour when loading is a commonly reported issue which presents risks to handlers and horse welfare. Existing literature and manufacturers recommendations suggests that alpha-casozepine may be effective in improving the behaviour and welfare of horses during loading onto a vehicle for transport. The current paper aims to assess the behavioural and physiological effects of a commercially available alpha-casozepine feed supplement (Zylkéne Equine) in horses during loading and confinement on a transport lorry. Subjects (n = 10) were loaded once with the supplement and once without, in a balanced random order with each subject acted as their own control. The handler was blind to treatment. Time to load onto the lorry, and movement of feet, licking and chewing, and vocalising within the lorry, were recorded as behavioural indicators of compliance and coping. Heart rate, heart rate variability, salivary cortisol, and infrared thermography of both core temperature and the discrepancy between eyes, were measured as indicators of arousal. There were no significant differences in physiology between Treatment and Control ($P > 0.05$). Treatment resulted in a significantly shorter Loading Time than control ($P = 0.04$), however, the actual difference in median time was only 0.45 seconds. No other behavioural indicator differed between Treatment and Control ($P > 0.05$). Power analysis revealed the sample was sufficient to detect a significant effect. Where modest effects were observed for a small number of variables, Treatment effect contradicted predictions. Taken together, this indicates that alpha-casozepine does not affect a horse's ability to cope with loading and confinement in a horse lorry. Further work is required to

ascertain whether the maximum dosage – twice that used here – might affect coping and behaviour in horses.

Keywords: alpha-casozepine; horse loading; infrared thermography; salivary cortisol; heart rate variability; stress

Introduction

Horses are routinely travelled for access to safe off-road riding, veterinary care, breeding, sale or moving to a new home environment. However, transport is a known stressor in horses (Schmidt et al., 2010) due to features such as confinement, novel noises, unstable flooring, the presence of unfamiliar conspecifics and sudden changes in light. For this reason, problem behaviour when loading is a commonly reported issue, which presents risks to handlers and horse welfare. During loading, the handler motivates an approximately 500kg animal with a highly evolved flight response into a confined space, which neither the horse nor handler can easily escape if an accident occurs. Sedation may be offered to improve behaviour but this may reduce the motor-control of the animal, increasing the risk of loss of balance during transport and subsequent injury. Further, sedatives are commonly banned in horses being transported for competition (FEI, 2018) and sedatives that mainly affect motor-control may make the horse more manageable and cause them to appear calmer, without addressing the underlying anxiety trigger by the environment. Ideally, correctly applied behaviour modification techniques aimed at habituating the horse and training them to respond to lead-rope pressure should be implemented, rather than the use of force (McGreevy and McLean, 2009). Such training aims to improve the horse's ability to tolerate a stressor, however, this process may still incur

risk to even experienced trainers. Additionally, it is possible that an animal may need to be transported at short notice, without the benefit of such training. Therefore, any practical solutions that may improve the efficacy of training, or limit the welfare impact of unavoidably stressful events, are warranted.

Dietary supplementation with alpha-casozepine is thought to have anxiolytic properties. Alpha-casozepine originates from S1 casein, a protein in cow's milk and fits into a segment of GABA-B receptors which are responsible for anxiolytic activity (Landsberg et al., 2017). Whilst research into this supplement is limited, McDonnell et al., (2014) found a significant improvement in horses' compliance and comfort during twelve routine healthcare and treatment procedures when supplemented with Zylkène Equine, a commercially available alpha-casozepine. This supported previous findings (McDonnell et al., 2013) which showed that semi-feral ponies treated with Zylkène whilst undergoing the process of initial training were more calm, compliant and progressed better than those not having Zylkène in their feed. This anxiolytic effect is also noted in rats (Miclo et al., 2001) and cats (Beata et al., 2007). Zylkéne Equine is suggested by the manufacturers for use in loading and transporting horses (Vetoquinol, 2018). Moreover, it is safe for use and not currently listed as banned for competition use (FEI, 2018). However, no studies to date have measured the physiological impact of such supplementation and compliance is not necessarily an appropriate indicator of coping in horses (Squibb et al., 2018), though it is highly desirable for handlers.

Physiological indicators of arousal can be measured non-invasively in a number of ways. Heart Rate Variability (HRV) is advantageous because it can be used to investigate the functioning of the autonomic nervous system, as variability decreases with an increase in stress (von Borell et al., 2007). Infrared thermography (IRT) on

ocular (eye) surface temperature has also been used in horses to monitor stress responses (Ijichi et al., 2018; Valera et al., 2012). It has been validated against cortisol (Valera et al., 2012) and can detect fear during novel object tests (Dai et al., 2015). Additionally, a discrepancy in temperature between the left and right eye may indicate hemispheric dominance indicative of affective state (Lush and Ijichi, 2018), though this requires further validation. Cortisol is released as a response to stressful events and can be measured from saliva samples (Yarnell et al., 2013). Studies based on blood plasma cortisol changes have repeatedly shown that transport is stressful for horses, however, blood sampling causes stress in itself (e.g. Fazio et al., 2008). As salivary cortisol is validated against blood samples (Peeters et al., 2011), salivary cortisol sampling is the best candidate for non-invasively sampling rapid changes in cortisol.

The current experiment aims to assess the effects of a commercially available feed supplement, Zylkéne Equine, on behaviour and physiology in horses during loading and confinement on a transport lorry. To this end, subjects were loaded once with the supplement and once without, in random treatment and subject order. Time to load onto the lorry, movement of feet, licking and chewing, and vocalising within the lorry were recorded as behavioural indicators of compliance and coping. Heart rate, heart rate variability, changes in salivary cortisol and infrared thermography of both core temperature and the temperature discrepancy between eyes, were measured as indicators of arousal. It was hypothesised that horses would load more quickly but move, vocalise, lick and chew less within the lorry in the treatment, compared to the control tests. It was also hypothesised that horses would have lower heart rate, higher heart rate variability, lower core temperature, more negative discrepancy scores and reduced cortisol changes in the treatment, compared to the control tests.

Materials and methods

Subjects

10 healthy horses (6 geldings and 4 mares) of mixed breeds and ages were tested between 26th March and 12th April 2018. Ages ranged from 8-25 years of age (mean = 12.6; IQR = 9.25-14.5). Horses were stabled at two private livery yards in Gloucestershire and were tested in their home environment to reduce the effect of environmental novelty. Subjects were travelled at least once a month as part of their normal management routine and had no known phobia to travelling. This restriction was imposed by Hartpury Unversity's ethics committee to ensure high animal welfare standards were met. Horses were managed at the discretion of their owners which meant that workload, turnout and feeding varied according to age and current use, as well as owner preferences.

Experimental Design

This was a within-individual experimental design with each subject acting as its own control. Each subject was loaded once with Zylkéne Equine and once without. The order of the treatments were randomly allocated. To counterbalance the study there were equal numbers of supplemented and control horses in each trial. This limited the possibility of a false positive due to habituation through repeated exposure (Hawson et al., 2010). Subject order within the group was pseudo-randomised to account for owner availability. The handler was blind to treatment to prevent any sub-conscious bias affecting handling and therefore subject responses. Tests were repeated 2 weeks apart at the same time of day ± 30 minutes. With the exception of

the test itself, subjects were managed as per their normal daily routine reducing the impact of differing management of each testing day. A wash-out period has not been established for this supplement by the manufacturer and so two weeks was used as an estimated generous wash-out period for subjects receiving Zylkéne in the first trial. This assumption was tested during data analysis (see 2.9 Statistical Analysis).

Feeding Protocol

Zylkène Equine was fed once daily for four days prior to testing, as per minimum dosage in the manufacturer's instructions (Vetoquinol, 2018). Horses weighing up to 500kg were fed 1000mg daily, while horses over 500kg were fed 2000mg of Zylkène Equine. Mean subject weight was 492.9kg (±70.34). The researcher met with the owners of the horses a week before the test was due to take place to provide the correct amount of Zylkène Equine supplement and ensure that the owner was clear about how the supplement must be fed. The supplement needed to be fed to the horses in their morning feeds to ensure that they received their final dose on the morning that the test took place. Prior to testing on treatment trials, the same researcher (S.G.) confirmed that the subject had been fed the supplement. Aside from the addition of the supplement for one trial, feeding was kept as per the subject's normal routine.

Handling and Loading

The current study used the same Equi-trek rear-facing 3.5t lorry for all tests. The internal divider was removed to allow the handler to move safely in the lorry with the

subject and to provide the subjects with more room to express behaviour (Figure 1). Subjects wore protective equipment such as rugs, travel boots or poll-guards at the discretion of their owner. All subjects were handled by the same individual (C.I.) who is experienced in loading horses and experimental handling and was blind to treatment. Horses were led to a marker 3.5m from the ramp of the lorry and halted. Horses were handled using appropriate pressure and release (McGreevy and McLean, 2009). Forward pressure on the leadrope was used to indicate the horse should step forward. This was immediately released when the horse complied. If the horse did not respond to leadrope pressure, they were rhythmically tapped on the rib-cage first with increasing speed and then increasing intensity if required, until they took a forward step. Soft vocal cues were also used to indicate correct responses and tactile positive reinforcement, including wither scratching (Thorbergson et al., 2016). This was used on loaded horses to encourage them to stand while the ramps were closed. Once inside the closed lorry, subjects were cross-tied in the center of the lorry with elasticated safety lines (Figure 1). The handler then took the post-loading IRT images before stepping through the internal door and sitting out of the subject's vision on a stool placed in the equipment compartment. Each horse remained within the lorry for 5 minutes, the doors were then re-opened and the subject unloaded.

Infrared Thermography

Using a FLIR E4 thermal imaging camera (FLIR Systems, USA.), the researcher took an image of both of the subject's eyes. The camera was held at approximately a

ninety-degree angle and 1m distance from the eye as accurately as possible within the confines of the space available. IRT readings were taken in the stable before testing (S.G.), once loaded onto the lorry when the ramp had been closed and before the ramp was opened and the horse was unloaded from the lorry (C.I.). The temperature was analysed for each horse retrospectively using FLIR tools (ver. 5.9.16284.1001). The maximum temperature within the palpebral fissure from the lateral commissure to the lacrimal caruncle (Yarnell et al., 2013) was used and the discrepancy between the temperatures for each eye was calculated by subtracting the temperature of the left eye from the right eye (Lush and Ijichi, 2018). C.I. and S.G. analysed the images independently and, on the rare instance where they varied, the highest recorded temperature for each image was used for analysis. The average of both eyes is referred to as Core Temperature. The difference in temperature between the eyes is referred to as Temperature Discrepancy.

HRV Readings

A Polar Equine V800 heart rate monitor (Polar Electro Oy, Kempele, Finland) was paired to an elasticated adjustable surcingle. This was fitted to each horse after IRT images were taken but prior to leaving the stable, by wetting the girth area and then ensuring close contact to ensure conductivity (S.G.). The paired watch was looped onto the surcingle to ensure that it remained within connectivity boundaries at all times. Subjects had a minimum of 5 minutes to habituate to the surcingle which was deemed to be sufficient as all subjects had previously worn girths and/or lunging rollers. Recordings began at a marker 3.5m meters from the ramp of the lorry and recorded continuously during loading, confinement and unloading. Recording was stopped when the horse returned to the marker after unloading.

Heart rate analysis was carried out by C.I. using Kubios HRV software (ver. 3.0.2 Biomedical Signal Analysis and Medical Imaging Group, Department of Applied Physics, University of Eastern Finland, Kuopio, Finland.). Kubios settings were adjusted in line with previous equine studies (Ille et al., 2014; Squibb et al., 2018). Specifically, artefact correction was set to custom level 0.3, thus removing RR levels varying by more than 30% from the previous interval. Therefore, where a single RR interval was more than 30% different from the preceding interval, it was deemed to be an incorrect reading. Trend components were adjusted using the concept of smoothness priors set at 500ms, to avoid the effect of outlying intervals. The STD RR value, being the standard deviation of RR intervals, was used as the HRV figure to reflect both short-term and long-term variation with the series of RR intervals. The root mean square of successive RR intervals (RMSSD value) was recorded as an indicator of vagal tone (Schmidt et al., 2010).

Cortisol Samples

Cortisol samples were taken using an Equisal saliva collection kit. The swab was removed from its packaging and inserted into the side of the horse's mouth through the interdental space, between the front and back teeth and above the tongue. The swab was moved gently around the top of the tongue until enough saliva was collected. This was judged using the colour change indicator, which turned from white to pink when sufficiently saturated. Once the sample collection was complete the swab was placed into a tube and chilled until it could be frozen, awaiting analysis.

Two saliva samples were taken, per horse, for each condition. The first sample was taken in the stable to determine a baseline level of cortisol for each horse by the same experimenter (S.G.). This was done after IRT readings – to ensure that the swabbing did not elevate core temperature - but before the heart rate monitor was fitted – which might affect cortisol in sensitive horses. The second saliva cortisol sample was taken after 5 minutes within the lorry, after the final IRT images were taken and before the subject was unloaded. The researcher (C.I) re-entered the horse compartment through the internal door and took the second sample in the same method described above. Pre-test cortisol values were subtracted from post-test values to indicate the change in cortisol as a result of loading and confinement (Table 1). This was to account for any variation in cortisol that was not the result of testing, such as slight diurnal differences or uncontrollable extraneous sources of stress. Baseline cortisol, post-test cortisol and changes in cortisol were included in further analysis.

Table 1. Baseline, post-test and change in salivary cortisol levels (μg/dL) for each subject in treatment and control trials.

	Treatment			Control		
Subject	**Baseline**	**Post-test**	**Change**	**Baseline**	**Post-test**	**Change**
1	0.3	0.4	0.1	0.19	0.26	0.07
2	0.14	0.09	-0.05	0.19	0.17	-0.02
3	0.08	0.101	0.021	0.15	0.13	-0.02
4	0.24	0.13	-0.11	0.12	0.11	-0.01
5	0.11	0.16	0.05	0.16	0.07	-0.09
6	0.05	0.07	0.02	0.12	0.09	-0.03
7	0.05	0.06	0.01	0.2	0.22	0.02
8	0.06	0.04	-0.02	0.08	0.04	-0.04
9	na	na	na	na	na	na
10	0.02	0.04	0.02	0.06	0.04	-0.02

Samples were analysed by S.G., K.S., A.C. and I.B. Saliva samples were kept within an ice cooler until transported to the laboratory where they were stored at -20 degrees until analysed. Samples were frozen on the day of sampling within approximately 4 hours of the saliva collection. To defrost swabs, all samples were stored at 4 ºC Samples were spun down using a centrifuge for approximately 5 minutes at full speed to extract the liquid.

When analysing, all reagents and the microtitre plate were brought to room temperature before starting the protocol. A 1X wash buffer, enough for the current day's requirement, was prepared.. Plate layout was determined with standards, controls and saliva samples assayed in duplicate. The protocol followed Salimetrics Assay (Salivary Cortisol ELISA kit) and was as follows:

24 mL of Assay Diluent was pipetted into the disposable tube. 25 µL of standards, controls, and saliva samples were pipetted into the appropriate wells. 25 µL of Assay Diluent was pipetted into 2 wells to serve as the zero. 25 µL of Assay Diluent was pipetted into each non-specific binding well. The Enzyme Conjugate was diluted

1:1600 by adding 15 µL of the conjugate to the 24 mL tube of Assay Diluent prepared earlier. The conjugate tube was centrifuged for approximately 5 minutes to bring the liquid down to the tube bottom. The diluted conjugate solution was mixed and 200 µL was added to each well. The plate was mixed on a plate rotator for 5 minutes at 500 rpm and incubated at room temperature for a total of 1 hour. The plate was washed 4 times with the 1X wash buffer. After each wash, the plate was thoroughly blotted on paper towel before it was turned upright. The plate was mixed again on a plate rotator for 5 minutes at 500 rpm and incubated in the dark (covered) at room temperature for an additional 25 minutes. 50 µL of Stop Solution was added to each well. The plate was mixed on a plate rotator for 3 minutes at 500 rpm. This was continued until all wells showed a yellow colour. The plate was read in a plate reader at 450 nm within 10 minutes of adding the Stop Solution.

Behavioural Observations

Researchers recording behaviour were blind to treatment. The time taken to load was measured by the same researcher (K.S.) using a stopwatch. Time was started when the handler stepped past the marker 3.5m from the ramp and ended when the final hind foot of the subject entered the lorry. Once inside the lorry, horse behaviour was recorded by a camera mounted on a tripod within the equipment compartment (C.I.). This recorded through the interior door between the equipment and horse compartments, which was secured in the open position (Figure 1). The recording began after the second IRT reading was taken and captured the 5 minute confinement period that followed.

Behaviour was recorded by the same researcher (C.I.) as individual instances for each variable. The number of times the subject moved their feet was recorded as an indication of frustration causing displaced locomotive behaviour and a failure to remain immobile (McGreevy and McLean, 2010). This included any instance where the foot was raised off the ground and included kicking, pawing and steps. The number of times the horse expressed licking and chewing behaviour was recorded. This included sideways movement of the jaw, accompanied by audible grinding, with or without the protrusion of the tongue. Although the ethological significance of licking and chewing is not yet fully understood, it is observed during potentially stressful circumstances (Krueger, 2007). Therefore, it was measured as a supplementary behavioural indicator. The number of vocalisations was recorded and characterised by audible neighing, separated by silence. Such vocalisations are used to regain contact with conspecifics (Houpt, 2001) and may indicate arousal caused by isolation within the lorry.

Statistical Analysis

Statistical analysis was carried out using R (R Development Core Team, 2017). The normality of the sampling distribution was tested using a Shapiro-Wilks test prior to tests of difference (Field et al., 2012). Paired T-tests or Wilcoxon ranked-sum tests were used to detect differences between Treatment and Control as appropriate for normality. Post-hoc effect sizes were calculated (Field et al., 2012; pp 393 & 665) to determine how meaningful changes in behaviour and physiology were. Power analysis was conducted on T-tests to determine whether non-significant differences were due to a lack of effect or insufficient sampling (Field et al., 2012).

Post-hoc tests of difference were conducted to determine whether an inadequate wash-out period may have confounded results, limiting the ability to detect a truly significant effect. If subjects were treated for the first trial, and the supplement had not completely washed out by the time they were tested for the Control trial, this may cause an insignificant effect in the whole sample, when in fact, the supplement is effective. Therefore, for variables that were not significantly affected by treatment in the whole sample, a subset of subjects who were tested with the control first (n = 5) were tested for differences between Treatment and Control. Subjects who were tested with the control first could not have had control trials affected by residual substance. Therefore, if this test of difference is significant, it indicates that non-significant findings in the whole sample were the result of residual supplementation and insufficient wash-out period. If the test is insignificant, it confirms non-significant results seen in the whole cohort.

Results

There were no significant differences in physiology between Treatment and Control (Table 2).

Table 2. Differences in physiological measures between Treatment (T) and Control (C). Paired T-tests (PT) state the mean, standard deviation (S.D.) and t-value (T) and statistical power. Wilcoxon Signed-Rank (W) tests state the median, inter-quartile range (IQR) and v-value (V). N = 10 for all tests, except cortisol (N = 9).

Variable	Test	Mean/ Median	S.D./IRQ	Test	V/ T	P	Effect Size	Power
Heart Rate (bpm)	T	78.02	21.42	W	39	0.28	-0.35	NA
	C	75.36	20.43					
Heart Rate Variability (ms)	T	73.08	±32.97	PT	0.65	0.53	0.21	0.79
	C	66.19	±32.37					
RMSSD (ms)	T	47.25	34.62	W	25	0.77	-0.09	NA
	C	44.65	30.38					
Baseline Core Temp. (ºC)	T	34.66	±1.17	PT	0.36	0.73	0.12	0.79
	C	34.37	±1.64					
Core Temp. Post-Loading (ºC)	T	34.86	±0.64	PT	0.39	0.7	0.02	0.79
	C	34.65	±1.48					
Core Temp. Post-Confinement (ºC)	T	34.95	±0.72	PT	0.8	0.45	0.11	0.82
	C	34.56	±1.06					
Baseline Temp. Discrepancy (ºC)	T	0.24	±0.8	PT	-1.32	0.22	0.24	0.92
	C	0.51	±1.1					
Temp Discrepancy Post-Loading (ºC)	T	-0.04	±0.58	PT	-1	0.34	0.15	0.87
	C	0.19	±0.55					
Temp. Discrepancy Post-Confinement (ºC)	T	0.3	0.33	W	30.5	0.76	-0.1	NA
	C	0.1	0.46					
Baseline Cortisol (µg/dL)	T	0.12	±0.1	PT	-0.84	0.42	0.28	0.71
	C	0.14	±0.05					
Post-Test Cortisol (µg/dL)	T	0.12	±0.11	PT	-0.14	0.89	0.05	0.89
	C	0.13	±0.08					
Change in Cortisol (µg/dL)	T	0.005	±0.06	PT	0.93	0.38	0.31	0.86
	C	-0.016	±0.04					

There was a significant difference in Loading Time, but no other behavioural indicator differed between Treatment and Control (Table 3).

Table 3. Differences in behavioural measures between Treatment (T) and Control (C). Paired T-tests (PT) state the mean, standard deviation (S.D.) and t-value (T) and statistical power. Wilcoxon Signed-Rank (W) tests state the median, inter-quartile range (IQR) and v-value (V). N = 10 for all tests

Variable	Test	Mean/Median	S.D./IRQ	Test	V/ T	P	Effect Size	Power
Loading Time (secs)	T	8.5	1.7	W	7	**0.04**	-0.66	NA
	C	8.95	7.83					
Licking & Chewing	T	8.5	8.73	W	23	0.65	-0.15	NA
	C	11	10.25					
Feet Movement	T	31.5	±34.56	PT	-0.92	0.38	0.29	0.85
	C	44.5	±44.67					
Vocalising	T	2.8	±2.8	PT	0.61	0.56	0.2	0.78
	C	2.3	±2.5					

There were no significant differences between Treatment and Control in subjects tested with Control before Treatment, with the exception of Core Temperature Post-Confinement (Table 4). Power was sufficient in all tests (Tables 2, 3 & 4).

Table 4. Differences in measures between horses tested under Control (C) conditions first and Treatment (T) second (n = 5). Paired T-tests (PT) state the mean, standard deviation (S.D.) and t-value (T) and statistical power. Wilcoxon Signed-Rank (W) tests state the median, inter-quartile range (IQR) and v-value (V).

Variable	Test	Mean/ Median	S.D./IRQ	Test	V/ T	P	Effect Size	Power
Heart Rate (bpm)	T	100.59	±33.3	PT	-1.24	0.28	0.53	0.93
	C	85.06	±15.59					
Heart Rate Variability (ms)	T	54.97	59.62	W	7	1	0	NA
	C	52.68	29.19					
RMSSD (ms)	T	72.34	±61.31	PT	-0.77	0.48	0.36	0.84
	C	55.5	±36.31					
Baseline Core Temp. (ºC)	T	35.0	0.3	W	2	0.19	-0.59	NA
	C	33.95	0.3					
Core Temp. Post-Loading (ºC)	T	34.95	0.85	W	1	0.13	-0.69	NA
	C	33.75	1.6					
Core Temp. Post-Confinement (ºC)	T	35.35	±0.33	PT	-2.76	**0.05**	0.81	0.99
	C	34.53	±0.35					
Baseline Temp. Discrepancy (ºC)	T	0.64	±0.95	PT	0.85	0.44	0.31	0.86
	C	0.96	±1.45					
Temp Discrepancy Post-Loading (ºC)	T	-0.1	0.3	W	3.5	0.58	-0.25	NA
	C	-0.1	0.3					
Temp. Discrepancy Post-Confinement (ºC)	T	0.22	±0.2	PT	-0.99	0.38	0.44	0.89
	C	0.02	±0.27					
Change in Cortisol (µg/dL)	T	0.04	±0.05	PT	2.07	0.13	0.78	0.98
	C	-0.02	±0.07					
Feet Movement	T	16	2	W	12	0.31	-0.45	NA
	C	10	14					
Licking & Chewing	T	17.6	±10.78	PT	-.057	0.6	0.27	0.80
	C	17.6	±20.04					
Vocalisation	T	3	3	W	3	0.18	-0.6	NA
	C	4	4					

Discussion

Problem behaviour is commonly seen during loading onto vehicles and anticipatory stress responses are seen in some horses in advance of transport (Schmidt et al., 2010). Supplementation with anti-anxiolytic substances may alleviate stress in this context and improve both horse welfare and handler safety due to improved behaviour (McDonnell et al., 2014). The current study aimed to determine the effects of alpha-casozepine supplementation on the behaviour and physiology of horses during loading and confinement in a horse lorry. Results indicate limited effects on behaviour at minimum dosage.

Supplementation with Zylkéne Equine had no significant effects on the physiological indicators examined. There was no difference in heart rate, heart rate variability, RMSSD, core temperature, discrepancy in eye temperature or salivary cortisol between Treatment and Control. Power analysis for all tests indicate that the sample size was adequate to detect an effect and therefore these results cannot be explained by limited sample size. The consistent lack of significant difference across all variables indicates that, at minimum recommended dosages, Zylkéne Equine was not effective in reducing anxiety or arousal in the current experiment. It is possible that the subjects in this experiment were not sufficiently aroused by the tests to differentiate between treatment and control as they had no known aversion to loading. On the contrary, if the substance has limited effect, efficacy may be further reduced in horses with a very pronounced anxiety response. Therefore, further testing on horses with known anxiety response to loading is required.

Interestingly, supplementation with Zylkéne Equine did have a significant and positive effect on time to load. Horses treated with alpha-casozepine loaded significantly faster into the lorry than when under control conditions. This result

370 cannot be explained by the handler biasing the loading procedure as this individual
371 was blind to the randomised treatment order. Whilst this is a positive indicator that
372 many horse owners would value, the actual difference in median time was only 0.45
373 of a second. This is arguably not a meaningful difference that handlers would value.
374 However, difference in loading time had a statistically strong effect. Therefore, a
375 more pronounced differentiation between the two treatments in horses that have
376 known reluctance to enter a transport vehicle may be possible. However, since the
377 supplement had no significant effect on physiology, this cannot be assumed. Without
378 altering the horse's affective state of arousal or stress, it is not clear how behaviour
379 would be meaningfully altered. In addition, McDonnell et al., (2014) noted little to no
380 effect of this supplement on loading time in their study. Within the current sample of
381 10 horses, it is possible that uncontrollable variations in mood or the environment
382 account for this difference. No behavioural variable other than time to load was
383 affected by supplementation. Instances of licking and chewing, vocalising, and
384 movement within the lorry were not significantly different between treatment and
385 control. Previous studies noted modest differences in behavioural indicators of stress
386 and compliance (McDonnell et al., 2014, 2013). However, these studies did not
387 utilise within individual differences and had small sample sizes, leaving them
388 vulnerable to the effects of individual differences.

389 The current study used a paired design which limits the confounding effects of
390 individual differences on results. One possible limitation of this approach is that
391 subjects who are tested with the Treatment first may have confounded Control tests
392 if a complete wash-out is not achieved. However, a sub-sample of subjects that
393 received Control before Treatment were analysed and most tests of difference were
394 not significant in this group. The only exception was core temperature post-

confinement which was significantly different in this sub-group. However, temperatures were significantly hotter in the treatment group, which does not support reduced arousal indicative of increased coping in subjects supplemented with Zylkéne Equine (Valera et al., 2012). Taken as a whole, this suggests that inadequate wash-out of the supplement does not explain the lack of effect noted in the current study.

The current study is not without limitations. For ethical considerations, only horses that were experienced travellers with no known aversion to loading were used and this may not reflect how the substance would act when used in anxious individuals. In particular, time taken to load may differ in subjects who find this aversive and increased arousal may differentiate between treatment and control. Further, the dosage was the minimum recommended by the manufacturer and manufacturer's guidelines are not sensitive to the body weight (Vetoquinol, 2018). Future work should test the substance at maximum recommended dosages which is approximately twice what was administered here. In addition, investigating the effects in subjects with a known aversion to loading is warranted.

Conclusions

In the current experiment, Zylkéne Equine had no significant effect on heart rate, heart rate variability, core temperature, discrepancy between eye temperatures or salivary cortisol. This indicates that this supplement does not affect a horse's ability to cope with loading and confinement in a horse lorry at the dosage used. These physiological indicators are supported by the behavioural indicators licking and chewing, feet movement, and vocalising when confined, which also did not differ

between treatments. However, horses did load significantly more quickly when supplemented with alpha-casozepine. Though it is important to note that the median difference was only 0.45 seconds and is therefore irrelevant. Further work is required to ascertain whether the maximum dosage – twice that used here – might affect coping and behaviour in horses. In addition, it is not clear whether the difference between Control and Treatment would be differentiated or attenuated by testing subjects with known anxiety responses during loading.

Acknowledgements

We sincerely thank our volunteers for their time and the use of their horses. Woodland Barn Farm, Cirencester and Fairfields Livery, Newent kindly allowed us to test horses on their premises. The authors of this manuscript have no conflict of interest to declare and no funding bodies to acknowledge.

Ethical Statement

Subjects took place following the informed written consent of the owner. The horses used in the sample were free from known injury or illness that would compromise welfare during testing and had transport experience. Subjects needed to have been transported at least once a month as part of their normal management routine. Subjects did not have any known phobia to travelling or the process of being transported (such as loading or unloading) that it would have been detrimental to their welfare.

After selection, horses were withdrawn from testing if a) the owner chose to withdraw the subject; b) C.I. deemed the horse physically or mentally unfit to continue, for

example, due to significantly increased HR on approaching the lorry; c) subjects took longer than 5 minutes to load. Horses were monitored constantly throughout the test via camcorder display screen by a researcher (C.I.) who remained in the lorry throughout the test. The test would be stopped immediately if a problem occurred or if the horse became overly stressed. If this situation occurred, the subject would be immediately removed and returned to their stable, though this did not occur.

Zylkène Equine is an extremely palatable, apple flavoured supplement which can be added to an existing diet. This ensured that there was no change to feeding or management practices. Additionally, there are no known side effects of Zylkène Equine and it is a product which is available 'over the counter' without a veterinary prescription. This supplement is safe to feed in conjunction with other therapies and in pregnant or lactating mares (Vetoquinol, 2018). There is no long term risk to the horse as this supplement is used short term, for the current study each horse required only four doses (the last day being the day of testing).

Although no side effects were expected to occur, horses were removed from the study if any adverse changes in behaviour were observed by the driver of the lorry, the owner or the researchers. Furthermore, the horses used in this study only took part with informed consent from their owners. The owners had the right to withdraw the horses from the trial at any point.

All data recorded during the experiment was solely for the purpose of the research described within the consent form and is only available to the researcher team. Any information personal to the subjects and their owners were kept discrete in compliance with The Data Protection Act 1998.

The authors of the current paper have no conflict of interest to declare.

Authorship Statement

The idea for this paper was conceived by Carrie Ijichi & Sophie Green; the study was designed by Carrie Ijichi and Sophie Green; the study was performed by Carrie Ijichi, Sophie Green, Keith Squibb, Aisling Carroll and Isobel Bannister; the data was analysed by Carrie Ijichi; the paper was written by Carrie Ijichi, Sophie Green and Aisling Carroll, the paper was edited by Keith Squibb and Isobel Bannister.

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